Culture conditions affect Lactobacillus reuteri DSM 17938 ability to perform glycerol bioconversion into 3-hydroxypropionic acid

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Lactobacillus reuteri, Plackett and Burman experimental design, growth medium composition, environmental culture conditions, glycerol bioconversion, 3-hydroxypropionic acid production yield

Abstract

The platform molecule 3-hydroxypropionic acid (3-HP) can be produced using *Lactobacillus reuteri* through a two-step bioprocess that involves a growth phase followed by a bioconversion phase. The bioproduction is performed by resting cells that convert glycerol into 3-HP and 1,3-propanediol in fed-batch mode. This work aimed at studying the effect of the growth conditions of *L. reuteri* DSM 17938 during the first step, on the glycerol bioconversion into 3-HP during the second step. A Plackett and Burman design was carried out to test, in controlled bioreactors, the effect of 11 growth conditions simultaneously, at fixed bioconversion conditions. The supplementation of the growth medium with vitamin B12 and cysteine displayed a negative effect on the 3-HP bioproduction. The addition of glucose, phytone peptone, Tween 80, 1,2-propanediol and betaine in the growth medium, together with a low temperature and an optimal pH of 6.0 during the growth phase increased the bioconversion duration from 56 h to 89 h at a glycerol feeding rate of 0.5 $g \cdot h^{-1}$. A validating experiment displayed that the 3-HP titer, 3-HP

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production yield and 3-HP specific production rate were significantly improved by 25 %, 150 % and 61 %, respectively.

Introduction

Using microorganisms to produce valuable biomolecules from renewable resources has gained great interest in the two last decades due to limitation of fossil fuel resources and overwhelming environmental issues. In such context, the development of a biotechnological process to produce the platform chemical 3-hydroxypropionic acid (3-HP) is a key issue as this weak organic acid displays many applications. Firstly, it can be used as preservative in the food or feed industry (1, 2). But its major attraction comes from its bi-functionality, i.e. the presence of a carboxyl group and a β-hydroxyl group, which makes 3-HP the precursor of a large variety of chemicals for multipurpose applications. It thus serves as a versatile agent for chemical reactions such as dehydration, oxidation, reduction, cyclization or polymerization that lead to the formation of acrylic acid, malonic acid, 1,3-PDO, propiolactone, valuable homopolymers (poly 3-HP) or copolymers (containing 3-HP), respectively (3). This explains why it was ranked as one of the top value-added platform molecules to be produced from biomass by the US Department of Energy (4). Some chemical synthesis routes have been proposed to obtain 3-HP from acrylic acid, 3-propiolactone, 3-hydroxypropionitrile, allyl alcohol, vinyl acetate and 1,3-PDO (5). However, these processes display several drawbacks such as the use of expensive raw materials, the high costs for operating processes and associated environmental issues (3).

Nowadays, a better alternative to produce 3-HP is based on biotechnological processes, by employing either natural microbial producers or genetically modified organisms (GMO) working as cell factories, including GMOs that do not produce 3-HP naturally and microorganisms producing 3-HP but genetically modified to improve their performances. Detailed information about genetically modified microorganisms is available in recent reviews

(6, 7). *L. reuteri* is an attractive candidate due to its safety characteristics, its probiotic properties and its anaerobic but aerotolerant attributes (8). In considering 3-HP bioproduction, *L. reuteri* is naturally capable of converting glycerol into 3-HP and 1,3-PDO in equimolar proportions, as it possesses all the enzymes of the metabolic pathway for 3-HP biosynthesis encoded by the *pdu* operon (9). It has the ability to synthesize vitamin B12 that is an essential co-factor for the first enzyme of the glycerol metabolism (10), but cannot use glycerol as a carbon source (11), which allows glycerol to be completely valorized into 3-HP and 1,3-PDO through the bioconversion pathway.

The 3-HP bioproduction has to be achieved through a two-step process, which includes a growth phase to produce a high cellular concentration and a second step to perform the glycerol bioconversion into 3-HP by resting cells (12). Glycerol is firstly converted to 3-hydroxypropionaldehyde (3-HPA) by a vitamin-B12 dependent glycerol dehydratase. 3-HPA is then either converted to 1,3-PDO or 3-HP through parallel reductive and oxidative branches that maintain the redox balance between NADH and NAD^+. 1,3-PDO is obtained from 3-HPA using the enzyme 1,3-propanediol oxidoreductase (13), while 3-HP is the product of three successive reactions using propionaldehyde dehydrogenase, phosphotransacylase and propionate kinase (12, 14). The conditions to be employed during the bioconversion step have been identified with the aim of increasing the 3-HP concentration and productivity. The use of fed-batch cultures with glycerol supply is required to prevent accumulation of the toxic intermediate 3-HPA, since the rate of 3-HPA formation is higher than that of its subsequent conversion (12). The maximum rate of glycerol supply to *L. reuteri* DSM 20016 has been determined at 0.75 $g \cdot h^{-1}$ to avoid 3-HPA accumulation (16). The use of micro-aerobiosis was recommended to improve the 3-HP bioproduction (17). Finally, the absence of glucose was shown to reduce 1,3-PDO production due to a redox cofactor imbalance (12) and to prevent the synthesis of undesirable fermentation products (i.e. lactic acid, ethanol, acetic acid, CO_2).

The influence of some conditions implemented during the growth step, prior to bioconversion, has been studied, in the vast majority of cases with the aim of improving the growth performances. By considering the nutritional requirements of *L. reuteri*, the influence of carbon and nitrogen sources has been determined. Different carbon sources have been used to trigger *L. reuteri* growth, such as glucose alone (18, 19), glucose plus fructose, saccharose (19), galactose, lactose, melibiose, raffinose, saccharose (20) and industrial wheat or sugar beet syrup by-products that contained glucose and fructose (21). From these studies, glucose was demonstrated as the better carbon source to maximize *L. reuteri* growth (21). The suggested concentrations of glucose were comprised between 20 $g \cdot L^{-1}$ (22) and 30 $g \cdot L^{-1}$ (21). As *L. reuteri* displays a heterofermentative metabolism, glucose is catabolized through two pathways (phosphoketolase (PK) and Embden-Meyerhof-Parnas (EMP) pathways) that operate simultaneously and lead to the production of lactic acid, acetic acid, ethanol, CO_2 and energy (19).

The effects of various nitrogen sources have been established on *L. reuteri* cell growth. Yeast extracts and peptones are commonly used for growth of lactic acid bacteria due to their composition in amino acids and vitamins which meets cell needs. Yeast extract is the most common source employed in the published studies concerning *L. reuteri* (21, 23). In addition, phytone peptone, which is obtained from papaic digestion of soybean meal, displays an interesting composition with 18 of the 20 natural amino acids (asparagine and glutamine are absent) and is a cheaper nutrient source than yeast extract. Phytone peptone was demonstrated to lead to the highest cell concentration by comparison with other nitrogen sources such as peptones of animal origin, tryptone, proteose peptone, tryptic soy broth, yeast extract, and beef extract, in *L. reuteri* DSM 20016 (24) and other *L. reuteri* strains (PTA-4965, 23272, and 55730) (25).

The addition of the amino acid cysteine was also considered as this component is an essential growth factor of some lactobacilli (26). For *L. reuteri* DSM 20016 and SD 2112 strains, it was

recommended at concentrations between 0.1 $g \cdot L^{-1}$ (24) to 0.5 $g \cdot L^{-1}$ (20). However, the addition of 2 $g \cdot L^{-1}$ cysteine in the culture medium was mentioned to decrease the production of vitamin B12 by *L. reuteri* JCM1112 by a factor 3 to 5 (10). This amino acid is also known as a potent reducing agent, thus displaying antioxidative properties that help the cells to cope with the oxidative stress encountered during the recovery and concentration steps, as demonstrated with *L. fermentum* (27).

Tween 80 is a key growth factor in culture media for lactobacilli, as it brings unsaturated fatty acids that allow reducing intracellular energy consumption, thanks to the down-regulation of de novo fatty acid synthesis in membrane phospholipids (28). It is for instance present at a concentration of 1 $g \cdot L^{-1}$ in MRS medium (12, 29) or added to reach 5 $g \cdot L^{-1}$ (21).

Besides, the influence of some other components in the culture medium of lactobacilli has been investigated. Supplementation with vitamin B12 (0.1 $mg \cdot L^{-1}$), as a mandatory cofactor for the first enzyme in the *pdu* pathway, although not influencing *L. reuteri* growth, positively impacts the 3-HP bioproduction (21). The addition of 1,2-propanediol (1,2-PDO) has been studied as it is involved in the activation of genes of the *pdu* pathway (16, 30). It was added at a concentration of 5 $g \cdot L^{-1}$ into *L. reuteri* DSM 20016 growth medium prior to glycerol conversion (14). Betaine is a methylated derivative of the amino acid glycine that acts as an osmolyte to help lactic acid bacteria counteract the osmotic shocks they face during the harvesting and concentration stages (31). By considering *L. buchneri* R1102, it was demonstrated that the combined supplementation with 2 $mmol \cdot L^{-1}$ betaine and 0.1 $mol \cdot L^{-1}$ KCl in the growth medium, together with a further addition of 0.6 $mol \cdot L^{-1}$ KCl at the end of growth, led to intracellular accumulation of betaine and improved cell survival during freeze-drying.

Formulating the environmental parameters (i.e. growth temperature, pH or the base used for pH control) to improve cell growth and further bioconversion is also challenging. The temperature for *L. reuteri* growth is generally established at 37 °C (18, 24, 32). However, the optimum temperature for growth may differ from that maximizing a given metabolic activity.

For example, cultures of *L. fermentum* CRL 251 and *Bifidobacterium longum* CRL 849 implemented at 30 °C showed a slower growth but a stimulated alpha-galactosidase activity, in comparison to that observed at 37 °C (33). In order to reduce inhibitory effects of acidic conditions, the pH must be controlled during growth with an appropriate base solution. The optimal pH for *L. reuteri* growth is generally between 6.0 and 6.8 (34). Recently, different pH values between 3.7 and 6.7 were tested during batch cultures of *L. reuteri* DSM 12246 and the final biomass concentration was enhanced at pH 5.5 (35). Finally, the use of two kinds of bases have been reported for pH control during *L. reuteri* growth phase: NaOH (36, 37) and NH_4OH (12, 38).

However, they have never been compared for bacterial growth or 3-HP bioproduction.

From this information, it can be seen that most previous works studied the effects of culture conditions on *L. reuteri* growth but not on 3-HP bioproduction. They show that many culture conditions during the growth phase affect the growth performances of *L. reuteri*. However the effects of these culture conditions were scarcely investigated on cell ability to subsequently convert glycerol into 3-HP. In addition, the reported studies focus on a limited number of factors, thus making it difficult to define the optimal conditions by taking all of them into account. In such context, the present work aimed at quantifying the effects of relevant culture conditions on *L. reuteri* capacity to produce 3-HP during the subsequent glycerol bioconversion stage. As 11 factors were selected, a Plackett and Burman experimental design was retained as it allowed identifying the key factors that act on 3-HP bioproduction and quantifying their main effects, together with reducing the number of experiments. All experiments were performed in bioreactors in order to evaluate the effects of the factors in well-controlled conditions.

Material and methods

Bacterial strain

149 The strain *L. reuteri* DSM 17938 was obtained from BioGaia AB, Stockholm, Sweden. It was
150 stored at -80 °C in cryotubes with glycerol 20 % as cryoprotectant. The inoculum was prepared
151 by cultivating the cells in MRS broth (Biokar Diagnostic, Beauvais, France) for 16 hours at 37
152 °C and 100 rpm and then in MRS broth supplemented with glucose 20 $g \cdot L^{-1}$ (VWR BDH
153 Prolabo, Leuven, Belgium) for 8 hours in the same conditions. Inoculation of the culture
154 medium was performed at a concentration of $5.1.10^{-6}$ g of cell dry weight (CDW) per liter
155 ($g_{CDW} \cdot L^{-1}$), corresponding to $5.8.10^{4}10^{3}$ cells$\cdot mL^{-1}$. The specific growth rate was calculated by
156 plotting the natural logarithm of CDW against time.

157 **Culture and bioconversion media**

158 The reference culture medium used for bacterial growth was composed of MRS broth added
159 with 20 $g \cdot L^{-1}$ glucose. In accordance with the experimental design, it was supplemented or not
160 with the following components: glucose 50 $g \cdot L^{-1}$ (instead of 20 $g \cdot L^{-1}$), yeast extract 25 $g \cdot L^{-1}$
161 (Organotechnie S.A.S, La Courneuve, France), phytone peptone 25 $g \cdot L^{-1}$ (Merck KGaA,
162 Darmstadt, Germany), Tween 80 4 $g \cdot L^{-1}$ (VWR), vitamin B12 0.1 $mg \cdot L^{-1}$ (Sigma-Aldrich,
163 Saint Louis, MO, USA), 1,2-PDO 3 $g \cdot L^{-1}$ (Sigma-Aldrich), cysteine 1 $g \cdot L^{-1}$ (Sigma-Aldrich),
164 betaine 0.234 $g \cdot L^{-1}$ (Sigma-Aldrich) together with KCl 7.455 $g \cdot L^{-1}$ (Prolabo, Fontenay-sous-
165 Bois, France). All media were autoclaved at 110 °C for 20 minutes. Additional glucose was
166 autoclaved separately to prevent its degradation due to the Maillard reaction (18).
167 The bioconversion medium was composed of sterile osmosis water into which a glycerol
168 (Sigma-Aldrich) solution at 100 $g \cdot L^{-1}$ was added at a constant flow rate (29).

169 **Two-stage process for 3-HP production**

170 The two steps of 3-HP bioproduction were carried out in controlled bioreactors. Cell growth
171 was conducted in batch mode in a 5-L Sartorius B bioreactor (Sartorius, Dourdan, France). The
172 agitation rate was set at 100 rpm thanks to two Rushton propellers. The temperature and pH
173 during growth were defined according to the experimental design (33 or 37 °C, pH 5.5 or 6.0).
174 The pH was controlled either with NH_4OH 14.8 $mol \cdot L^{-1}$ (Sigma-Aldrich) or with NaOH 8.75

mol·L^{-1} (VWR). The growth step was stopped when the cells reached stationary phase. Bacteria were harvested by centrifugation (Avanti® J-E centrifuge; Beckman Coulter, Fullerton, CA) at 6,200 *g* for 10 min at 4 °C. Cell pellets were then suspended in the same volume of sterile osmosis water to reach about 26.7 ± 9.5 g_{CDW}·L^{-1}, corresponding to about $3.2.10^{10} \pm 1.3.10^{10}$ cells·mL^{-1}.

Bioconversion was achieved in a 2-L bioreactor (Sétric Génie Industriel, Toulouse, France) with an initial working volume of 0.8 L. It was performed in fed-batch mode, defined by a constant glycerol feeding rate of 0.5 g·h^{-1} to avoid 3-HPA accumulation. The pH was controlled at 6.0 by addition of NH_4OH 1.48 mol·L^{-1}, the temperature was set at 37 °C and the agitation rate was maintained at 100 rpm with two Rushton propellers. Bioconversion was stopped when no more 3-HP was produced, which was evaluated by the stop of NH_4OH consumption.

Evaluation of cell concentration and analysis of cell physiological state by flow cytometry

The bacterial cell concentration was determined indirectly by measuring optical density at 600 nm (OD_{600}) (spectrophotometer UV-Vis Evolution™ 201, Fisher Scientific SAS, Illkirch, France). Then, bacterial concentrations expressed in CDW were obtained from OD_{600} values using the correlation: $CDW = 0.2639 \times OD_{600}$ ($R^2 = 0.99$).

The physiological state of bacterial cells was assessed by flow cytometry after double fluorescent staining with carboxyfluorescein diacetate (cFDA) and propidium iodide (PI) (39). The cFDA allowed assessing cellular enzymatic activity after internalization and cleavage by intracellular esterases, thus forming green fluorescent carboxyfluorescein (cF). Nucleic acid dye PI was used to characterize cells with damaged membranes, in which it formed a fluorescent DNA-complex. Samples were first diluted with McIlvaine buffer pH 7.3 (0.2 mol·L^{-1} disodium dihydrogen phosphate (J. T. Baker, Deventer, NL) and 0.1 mol·L^{-1} citric acid (Fisher Chemical, Elancourt, France)) to reach a cell concentration of 10^6 cells·mL^{-1}. One milliliter of diluted sample was added simultaneously with 10 µL cFDA (Chemchrom V8,

Biomérieux, Marcy-l'Etoile, France) diluted at 100 $mL \cdot L^{-1}$ in acetone (Fisher Scientific, Leicestershire, UK) and 10 µL PI (1 $g \cdot L^{-1}$ in distilled water, Sigma-Aldrich) before incubation at 40 °C for 10 min. Flow cytometry analyses were performed with a BactiFlow cytometer (Sysmex Partec, Roissy, France) equipped with the Flowmax software (Partec). Cell fluorescence was measured at 530 nm for cF and 670 nm for PI after excitation by a 488-nm emitting laser. Cells emitting in green only (530 nm) were considered as viable, while cells emitting in red only (670 nm) were considered as dead. The data collected included cell concentrations (in $cells \cdot mL^{-1}$) and percentages of viable and dead cells (29, 39).

Quantification of substrates and metabolites

Substrates used for growth (glucose) and bioconversion (glycerol) as well as growth products (lactic acid, ethanol and acetic acid) and bioconversion metabolites (3-HP, 1,3-PDO and 3-HPA) were quantified by high-performance liquid chromatography (Waters Associates, Molsheim, France). Growth samples were diluted twice with trichloroacetic acid (Sigma-Aldrich) at 60 $g \cdot L^{-1}$, then centrifuged at 13,000 *g* for 10 min at 4 °C before filtration through a 0.22 µm pore-size filter (Sartorius Stedim Biotech, Gottingen, Germany). Bioconversion samples were added with citric acid 5 $g \cdot L^{-1}$, centrifuged and filtered in the same conditions. For each sample, a volume of 20 µL was injected (Waters 717 plus) in mobile phase (H_2SO_4, Sigma-Aldrich) on a cation-exchange column Aminex HPX-87H (300 mm × 7.8 mm, Biorad, Richmond, USA). Two sets of conditions including column temperature, H_2SO_4 concentration and flow rate of the mobile phase, were applied to separate the components properly (Table 1). Detection was done by a refractive index detector (Waters 2414) and an UV detector (Waters 2489). Quantification was achieved using the Empower software (Waters Associates) with the help of external standards. All analyses were duplicated and concentrations are given in $g \cdot L^{-1}$.

224 **Table 1. Conditions of HPLC analyses**

	Condition A	Condition B
Temperature (°C)	35	60
H_2SO_4 concentration in mobile phase ($mmol \cdot L^{-1}$)	5	0.5
Flow rate of mobile phase ($mL \cdot min^{-1}$)	0.6	0.4
Compounds detected in growth samples (and corresponding retention times, in min)	Glucose (9.3), lactic acid (13.2), acetic acid (15.6), ethanol (21.2)	
Compounds detected in bioconversion samples (and corresponding retention times, in min)	3-HPA (14.9), 1,3-PDO (17.5), acetic acid (15.6), ethanol (21.2) (the two latter remaining from growth)	Lactic acid (18.7, remaining from growth), 3-HP (19.7), glycerol (20.7)

225

226 ## Assessment of carbon mass balance

227 The carbon mass balance has been calculated during growth (CMB_G, in % $mol \cdot mol^{-1}$)
228 according to the central metabolism of *L. reuteri* via PK and EMP pathways and during
229 bioconversion (CMB_B, in % $mol \cdot mol^{-1}$), from the equations below:

230 $$CMB_G = (3.n_{Lactic\ acid} + 2.n_{Ethanol} + 2.n_{Acetic\ acid} + n_{CO_2} + n_{C\ in\ Biomass}) / 6.n_{Glucose} \quad \text{(eq. 1)}$$

231 $$CMB_B = (n_{3\text{-}HP} + n_{1,3\text{-}PDO} + n_{3\text{-}HPA}) / n_{Glycerol} \quad \text{(eq. 2)}$$

232 With n being the number of moles of consumed glucose ($n_{Glucose}$) and glycerol ($n_{Glycerol}$),
233 produced lactic acid ($n_{Lactic\ acid}$), ethanol ($n_{Ethanol}$), acetic acid ($n_{Acetic\ acid}$), CO_2 (n_{CO_2}
234 (hypothesized to be equal to the number of moles of produced [ethanol + acetate])), carbon in
235 cells produced during growth ($n_{C\ in\ Biomass}$), 3-HP ($n_{3\text{-}HP}$), 1,3-PDO ($n_{1,3\text{-}PDO}$) and 3-HPA ($n_{3\text{-}HPA}$).

237 ## Experimental design and statistical analyses

238 A Plackett and Burman matrix (40) allowed creating an experimental design to test the effects
239 of 11 medium components and culture conditions on growth and subsequent 3-HP
240 bioproduction by *L. reuteri* DSM 17938. The general form of the model is given as follows:

$$Y_i = k_i + \Sigma\, a_i \cdot Y_{(+)i} \qquad \text{(eq. 3)}$$

Where Y_i is the response variable; k_i, the constant value of Y_i when all 11 variables are at their Minus levels; a_i, the linear coefficient of each variable to express the effect of the Plus level; and $Y_{(+)i}$, the value of the Plus level of each variable i.

The multiple regression analysis was performed using the XLSTAT software (Paris, France). The 11 experimental factors tested in this study are listed in Table 2 that specifies the concentrations or values characterizing the Minus and Plus levels in the experimental design. The conditions used in each experiment are listed in Table S1. They were randomized and the reference condition was performed in triplicate. The final cell concentration ($g_{CDW} \cdot L^{-1}$) was selected as the response variable to describe the effect of the factors on the growth step. The percentage of viable cells at the beginning of the bioconversion stage was included in the analysis. Three response variables were chosen to characterize the effect of the experimental factors on the bioconversion performance: total quantity of 3-HP produced at the end of the fed-batch bioconversion ($g_{3\text{-HP}}$), 3-HP production yield ($g_{3\text{-HP}} \cdot g_{CDW}^{-1}$) and duration of the bioconversion (h).

Table 2. Factors included in the Plackett-Burman experimental design to study the effects of culture conditions of *L. reuteri* DSM 17938 on its bioconversion performances

Medium components	**Minus level**	**Plus level**
Additional glucose ($g \cdot L^{-1}$)	20	50
Additional yeast extract ($g \cdot L^{-1}$)	0	25
Phytone peptone ($g \cdot L^{-1}$)	0	25
Tween 80 ($g \cdot L^{-1}$)	1	5
Vitamin B12 ($mg \cdot L^{-1}$)	0	0.1
1,2-propanediol (3 $g \cdot L^{-1}$)	0	3
Cysteine ($g \cdot L^{-1}$)	0	1
Betaine ($g \cdot L^{-1}$) and	0	0.234
KCl ($g \cdot L^{-1}$)	0	0.745
Culture conditions		
Temperature (°C)	33	37
pH	5.5	6.0
Base type (and concentration, in $mol \cdot L^{-1}$)	NH_4OH (14.8)	NaOH (8.75)

Results and Discussion

Kinetics of cell growth and glycerol bioconversion in the reference condition of the experimental design

In order to assess the kinetics of *L. reuteri* DSM 17938 in the reference condition, three replicates were performed. The experimental conditions are summarized with the code T1 in the Table S1. Figure 1 shows the cell growth kinetics during the first phase, whereas Figure 2 displays the bioconversion kinetics of during the second step.

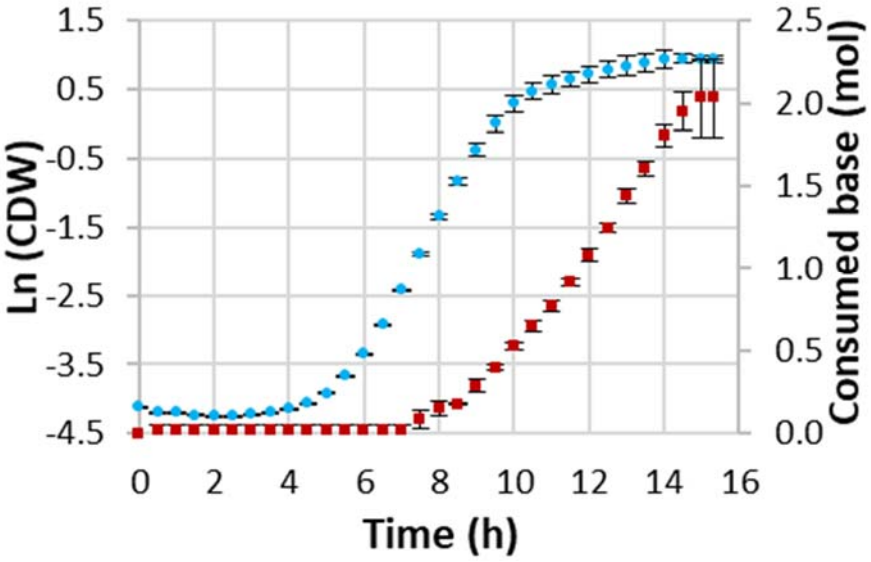


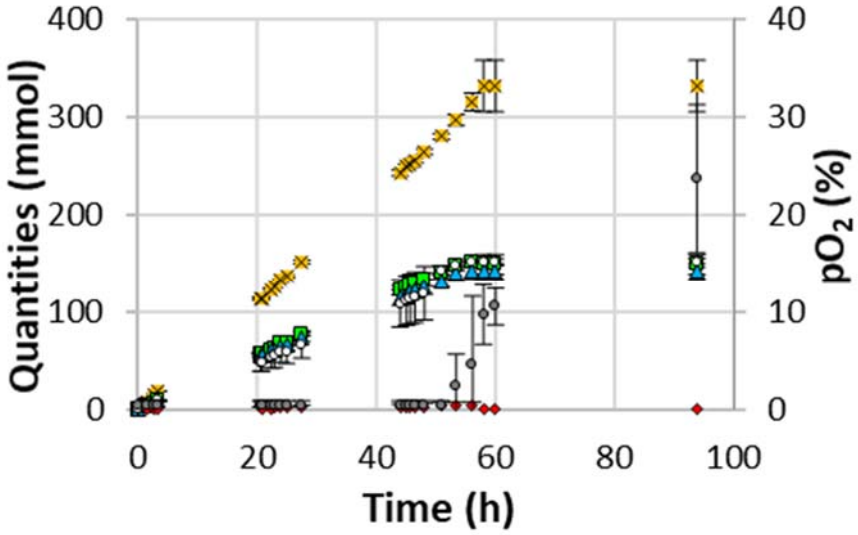
Figure 1. Kinetics of *L. reuteri* DSM 17938 growth and acidification in the reference condition

Blue circle: cell dry weight (CDW) expressed in $g_{CDW} \cdot L^{-1}$; red square: consumed base; error bars correspond to standard errors from 3 replicates.

From Figure 1, two successive exponential growth phases were observed, with different specific growth rates that were calculated at 0.99 (± 0.002) h^{-1} from 6 h to 9.5 h, and then 0.13 (± 0.01) h^{-1}, between 10.5 h and 14 h, indicating that a substrate limitation occurred. This limitation cannot be due to a lack of carbon source as the glucose concentration was equal to 8.5 (± 1.7) $g \cdot L^{-1}$ at 10 h. It may be caused by a lack of another nutrient source that was not searched in this study or by an inhibition of bacterial growth by the accumulation of lactic acid (41) and ethanol (42) that reached 4.6 (± 0.9) and 2.0 (± 0.4) $g \cdot L^{-1}$, respectively after 10 h. Meanwhile, no limitation was observed on organic acid production since the base was consumed regularly during the logarithm phase. However, when no more glucose was available in the medium, the base consumption stopped suddenly, together with growth, indicating that no more organic acid was synthesized. These results were confirmed by HPLC analysis. The base profile was then used to precisely detect the growth stop. Quantitative results obtained within the three reference experiments are summarized with the codes T1a, T1b and T1c in Table S2. The carbon mass balance during growth was verified at 104.33 (± 3.82) % for all the

experiments performed. This value was slightly higher than 100%, which could be explained by the fact that some carbon used for the growth may come from other sources than glucose (for instance yeast extract) that could not be quantified. A final CDW value of 3.3 (± 0.3) $g_{CDW} \cdot L^{-1}$ was achieved, which was similar to previous results obtained with the same *L. reuteri* strain at pH 5.5 (3.1 $g_{CDW} \cdot L^{-1}$) (12). From HPLC analyses, the presence of lactic acid (16.4 (± 1.2) $g \cdot L^{-1}$), acetic acid (3.7 (± 0.4) $g \cdot L^{-1}$) and ethanol (7.6 (± 0.8) $g \cdot L^{-1}$) was detected. The molar ratio of the sum of (ethanol + acetic acid) to lactic acid was close to 1 (0.97 (± 0.05) $mol \cdot mol^{-1}$). This value indicated that the PK pathway was prevalent in this heterofermentative *L. reuteri* strain, as previously observed by Burgé et al. (18). This value indicated that the PK pathway was prevalent in this heterofermentative *L. reuteri* strain, as previously observed by Burgé et al. (2015) (18). This result, obtained at controlled pH, is consistent with previous studies on *L. reuteri* ATCC 55730 (19) and *L. reuteri* DSM 17938 grown in free-pH conditions (18). Regarding the efficiency of ATP synthesis, the domination of PK pathway is unfavorable compared to EMP pathway (production of 1 instead of 2 ATP). Nevertheless, by producing less organic acid, the PK pathway reduced the inhibition of the bacteria by lactic acid, thus improving their survival in an acidic environment (18).

Kinetics of glycerol bioconversion into 3-HP and 1,3-PDO are illustrated in Figure 2. The carbon mass balance during bioconversion was verified at 100.12 (± 4.39) %. This step was performed using resting cells of *L. reuteri*, to allow the NAD^+ regeneration through 3-HP production in place of lactate production. The glycerol was fed into the bioreactor at a constant feeding rate 0.5 $g_{glycerol} \cdot h^{-1}$ until the bioconversion stopped. The slope of glycerol consumption was constant (Figure 2), which indicated that all substrate was consumed as and when it was supplied. The glycerol consumption ceased at the same time as the base consumption, which was confirmed from HPLC analyses. This indicated that the base profile was a relevant indicator of the cessation of the bioconversion.



311

312 **Figure 2. Kinetics of glycerol bioconversion into 3-HP and 1,3-PDO by *L. reuteri* DSM**
313 **17938 in the reference condition**
314 Yellow square with cross: consumed glycerol; green square: 3-HP; blue triangle: 1,3-PDO;
315 red diamond: 3-HPA; white circle: consumed NH_4OH; grey circle: dissolved O_2; error bars
316 correspond to standard errors from 3 replicates.
317

318 After 55.8 (± 2.2) h of bioconversion, two final products 3-HP and 1,3-PDO were obtained at
319 final concentrations of 11.8 (± 0.5) $g \cdot L^{-1}$ and 9.5 (± 0.5) $g \cdot L^{-1}$, respectively. In comparison to
320 the previous study of Dishisha et al. (2015) (16) using *L. reuteri* DSM 20016, the duration of
321 bioconversion was similar (56 h compared to 58 h), but the final 3-HP titer was 16 % lower
322 which was explained by the lower substrate feeding rate used in our study (0.5 $g_{glycerol} \cdot h^{-1}$
323 compared to 0.75 $g_{glycerol} \cdot h^{-1}$). The maximal 3-HPA content detected into the bioconversion
324 broth was equal to 0.4 ± 0.1 $g \cdot L^{-1}$. This value was about 10 times lower than the minimum
325 inhibition concentration reported for *L. reuteri* DSM 20016 (2.2 – 3.7 $g \cdot L^{-1}$) (43), thus
326 protecting it from detrimental effects (44). 3-HP was produced equimolarly to 1,3-PDO, as the
327 molar ratio was equal to 1.04 ± 0.06 $mol \cdot mol^{-1}$, which indicated that the redox balance between
328 NAD^+ and NADH was well maintained during the glycerol bioconversion. These results were
329 close to those obtained by (12) who obtained a molar ratio of 1 $mol \cdot mol^{-1}$. The molar ratio
330 between 3-HP and NH_4OH was equal to 1.08 ± 0.01 $mol \cdot mol^{-1}$, which is a little bit higher than

1. As no other organic acid was detected from HPLC analyses, this small divergence can be explained by a slight volatilization of the ammonia solution during the bioconversion. The total amount of 3-HP and 1,3-PDO produced at the end of the fed-batches were respectively equal to 13.4 ± 0.9 g and 10.8 ± 0.7 g. These values were lower than those obtained by (16) due to the lower supply rate of glycerol as aforementioned. The 3-HP production yield was equal to 0.78 ± 0.11 $g_{3\text{-}HP}.g_{CDW}^{-1}$, which was also lower than the higher value reported by (16).

Micro-aerobic conditions were applied in this study to facilitate glycerol metabolism toward 3-HP production (17). The partial pressure of dissolved oxygen (pO_2) in the medium remained at a value lower than 0.5 % until 51 h (Figure 2). At that time, the base consumption started to decrease, 3-HP and 1,3-PDO production ceased and pO_2 increased. This link between the pO_2 increase and the cessation of bioconversion is consistent with previous works (17, 45). This pO_2 increase may be due to a reduction of the activity of the enzyme NAD(P)H oxidase that uses molecular oxygen as a substrate (45). We may hypothesize that the stopping of the bioconversion corresponded to a global dysfunction of the whole enzyme pool of bacterial cells. In that case, the catalytic work of the NAD(P)H oxidase stopped concomitantly with that of the enzymes of the *pdu* operon that drives the 3-HP bioproduction. The decrease in oxygen consumption is thus observed simultaneously to the reduction in 3-HP bioproduction. Moreover, our results indicate that the pO_2 measurement may constitute an early on-line indicator of the cessation of bioconversion, which is an original approach for controlling the fed-batch bioconversion of glycerol into 3-HP.

Effect of culture conditions on *Lactobacillus reuteri* DSM 17938 growth performances

According to the Plackett and Burman experimental design (Table S1), various growth medium recipes and culture conditions were designed to screen, among 11 factors, the best combination to improve bacterial growth. The final biomass concentration ($g_{CDW}\cdot L^{-1}$) was retained as the variable to characterize the bacterial growth performance. Indeed, as the growth occurred during night without being monitored by a biomass probe, the effect of the conditions on the

Table 3. Effect of growth conditions on cell concentration and on glycerol bioconversion performances of *L. reuteri* DSM 17938

	Cell concentration at the end of growth ($g_{CDW} \cdot L^{-1}$)	Total quantity of 3-HP produced (g)	Bioconversion duration (h)	3-HP production yield ($g_{3\text{-}HP} \cdot g_{CDW}^{-1}$)
Constant	3.90	14.12	64.1	0.75
Additional glucose	0.58**	ns	4.0**	-0.23**
Additional yeast extract	ns	ns	ns	ns
Phytone peptone	ns	0.94*	5.7**	0.11*
Tween 80	ns	0.84*	2.1*	0.10*
Vitamin B12	ns	-2.16**	-10.6***	-0.15**
1,2-propanediol	ns	1.03*	4.5**	0.093*
Cysteine	ns	ns	ns	-0.10*
Betaine and KCl	ns	ns	4.6**	0.10*
Temperature	0.53**	ns	2.1*	-0.15**
pH	ns	ns	ns	0.096*
Base	ns	ns	ns	ns
R^2	0.873	0.893	0.978	0.919

Confidence level: * 90 %; ** 95 %; *** 99 %; ns: not significant; R^2 adjusted for degree of freedom.

specific growth rate could not be established. The results obtained from the 14 experiments are summarized in Table S2 and were used for statistical analyses. From Table 3, only two factors displayed a significant effect on *L. reuteri* DSM 17938 final concentration: the addition of glucose and the higher temperature, which positively influenced the final biomass concentration, by 15 % and 20 %, respectively. This result was confirmed by considering the number of viable cells at the end of the culture, enumerated by flow cytometry. It was improved by 34 % in the presence of 50 $g \cdot L^{-1}$ glucose, which matches with the aforementioned result. This positive effect of glucose on *L. reuteri* growth is consistent with early studies (18, 19, 21). Conversely, the addition of yeast extract or phytone peptone to the MRS medium as nitrogen sources did not improved neither the biomass concentration nor the cell viability. This result is contrary to those obtained in previous studies (21, 24) and suggested that the growth limitation previously observed in the growth phase cannot be justified by a lack of these nutrients. The existence of interactions between factors, which cannot be considered by the type of experimental design used in our study, can partly explain this observation.

Tween 80 and cysteine are both growth factors for lactobacilli. However, the current supplementation of these compounds at 5 $g \cdot L^{-1}$ and 1 $g \cdot L^{-1}$ respectively, seemed not enough to increase the cell concentration. The addition of vitamin B12, 1,2-PDO and betaine together with KCl did not affect the growth, which was expected as they were added to act during the bioconversion stage.

The temperature positively affected the biomass concentration that was higher at 37 °C than at 33 °C, which was confirmed by flow cytometry measurements ($5.6.10^9$ $cell \cdot mL^{-1}$ instead of $4.5.10^9$ $cell \cdot mL^{-1}$). This result is consistent with the conditions found in the digestive tract, the ecological niche of *L. reuteri* (46).

Finally, the pH value (pH 5.5 or pH 6.0) and the base used for pH control (NH_4OH or NaOH) did not significantly modify the final cell concentration of *L. reuteri* DSM 17938. Therefore, they can be used equally to maintain the pH during the growth phase.

Effect of culture conditions on *L. reuteri* DSM 17938 ability to convert glycerol into 3-HP

The Plackett and Burman experimental design allowed identifying the growth medium composition and culture conditions that affected the ability of *L. reuteri* DSM 17938 to further perform glycerol bioconversion into 3-HP. During all experiments, no residual glycerol was detected in the bioconversion broth. Table 3 summarizes the results obtained from the 14 different experiments (including three replicates of the reference condition), to explain the effects of the 11 factors on three variables that characterized the bioconversion step: quantity of 3-HP produced, bioconversion duration and 3-HP production yield. From Table 3, nine factors displayed a statistically significant effect on these variables. Only two factors did not show any significant effect on the characteristic variables of the 3-HP production step: the addition of yeast extract and the type of base used for pH control. The three variables were positively influenced by the addition of phytone peptone, Tween 80 and 1,2-PDO but negatively by a vitamin B12 supplementation. A high glucose concentration and a high temperature positively affected the bioconversion duration but negatively the 3-HP production yield. The addition of betaine enhanced the bioconversion duration and the 3-HP production yield. Finally, the addition of cysteine and a low pH value negatively influenced the 3-HP production yield. In addition to these three variables, the quantity of 1,3-PDO produced and the molar ratio between 3-HP and 1,3-PDO were also determined and will be analyzed when required.

As explained above, the growth medium supplementation with a higher glucose concentration led to a higher quantity of harvested biomass and a higher percentage of viable cells at the beginning of bioconversion. However, in the experimental conditions chosen during the bioconversion step, no increase of 3-HP production was observed, which is explained by the moderate glycerol feeding rate (0.5 $g_{glycerol} \cdot h^{-1}$) used in this study. In other words, the quantity of glycerol provided was insufficient to feed the higher cell quantity obtained in this condition.

As a consequence, the specific feeding rate of glycerol was lower in the condition of the higher level of glucose addition (18.1 ± 3.5 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$) as compared to that observed with the lower level of glucose addition (32.9 ± 8.6 $mg_{glycerol} \cdot g_{CDW}^{-1} \cdot h^{-1}$). This observation was supported by the decrease of the 3-HP production yield (- 30.7 %). The longer duration of bioconversion (+ 6.2 %) noticed when the cells were previously cultivated in the presence of a higher glucose concentration might be explained by a better enzymatic activity of the total bacterial population at the beginning of bioconversion stage, as observed by cF fluorescence measurements. This better activity allowed the cells to convert glycerol for a longer time.

Results showed that phytone peptone slightly promoted the 3-HP bioproduction whereas yeast extract did not (Table 3). As these two compounds act as amino acid sources, the positive effect of phytone peptone can be related to a specific amino acid composition that differs from that of yeast extract. From the information available from the suppliers, phytone peptone contained more arginine, histidine, tyrosine (+ 200 %) and glycine (+ 90 %) than yeast extract. Particularly, the amino acids arginine and glycine have been shown to be used for energy production and were linked to the survival of *Lactococcus lactis* under starvation (47) and *Lactobacillus sakei* under acidic conditions (48).

A positive effect of Tween 80 supplementation was observed on cell ability to produce 3-HP (Table 3) that is consistent with (21). This component is known to support cell division of lactobacilli by modifying the cell membrane fatty acids composition (49). In addition, it induces a down-regulation of the *de novo* fatty acid synthesis that helps the cells to save intracellular energy (28).

Addition of 1,2-PDO significantly improved the three variables characterizing the bioconversion step (Table 3), in agreement with (16). Here we consider that due to the structural similarity between glycerol and 1,2-PDO, the addition of the latter in the growth medium might help the cells to prepare their enzymatic machinery.

A negative effect of the addition of 0.1 $mg \cdot L^{-1}$ vitamin B12 in the growth medium was observed on the quantity of 3-HP produced, the bioconversion duration and the 3-HP production yield (Table 3). This result was an unintended outcome because this compound is a required cofactor of the first enzyme of the *pdu* metabolic pathway (12). It was reported that a supplementation of vitamin B12 at 0.1 $mg \cdot L^{-1}$ in the bioconversion medium showed a positive impact on 3-HP production by *L. reuteri* DSM 17938 (21). Thus, it could be hypothesized that the early addition of exogenous vitamin B12 in the growth medium may reduce the ability of the cells to produced endogenous vitamin B12 during the further step of bioconversion.

The addition of betaine together with KCl led to a longer bioconversion, together with a higher production yield (Table 3) that can be explained by the protective effect of betaine to osmotic stress (50). This result can be linked to that of (31) who related a higher intracellular betaine content to a more rigid membrane that led to the reduction of water exchange between the intracellular and extracellular compartments in *L. buchneri* R1102 (31).

The addition of cysteine was related to a reduction of the 3-HP production yield (Table 3). This observation can be explained by its negative effect on vitamin B12 biosynthesis by *L. reuteri*, as previously demonstrated (10). As another hypothesis, cysteine may counteract the oxygen utilization by the cells, as suggested by (27).

Regarding the influence of the growth temperature on the further bioconversion step, a higher 3-HP production yield but shorter bioconversion duration was observed when the growth was conducted at 33 °C instead of 37 °C (Table 3). As the glycerol feeding rate was fixed at a moderate value of 0.5 $g_{glycerol} \cdot h^{-1}$ during bioconversion, this higher 3-HP production yield was the consequence of the lower cell concentration achieved at the end of the growth phase.

The pH value during growth had a very little effect on the bioconversion step. Only the 3-HP production yield decreased slightly when the pH was reduced from 6.0 to 5.5 (Table 3). This difference could be ascribed to a small, even not significant, increase in the final biomass concentration when the growth was conducted at pH 5.5. Finally, it was demonstrated that the

type of base used had no effect, thus allowing us to use them indiscriminately. However, as the base NH_4OH prevents the formation of salt in the medium, this base might be preferred to facilitate further downstream processes.

Selected growth conditions that improve *L. reuteri* DSM 17938 ability to convert glycerol into 3-HP

In order to enhance the capacity of *L. reuteri* DSM 17938 to perform 3-HP bioproduction at a glycerol feeding rate of 0.5 $g_{glycerol} \cdot h^{-1}$, the growth conditions leading to the best 3-HP titer, bioconversion duration and 3-HP production yield have been identified. They consist in MRS medium added with glucose (20 $g \cdot L^{-1}$), phytone peptone (25 $g \cdot L^{-1}$), Tween 80 (4 $g \cdot L^{-1}$), 1,2-PDO (3 $g \cdot L^{-1}$), betaine (0.234 $g \cdot L^{-1}$) plus KCl (0.745 $g \cdot L^{-1}$), temperature (33 °C) and pH 6.0. No additional yeast extract, no supplementation with vitamin B12 nor cysteine were required, and the base to control the pH was likely unimportant. This set of conditions has been assayed to demonstrate its positive effects on the bioconversion performances. In comparison to the reference condition, the 3-HP titer was increased from 11.8 ± 0.5 $g \cdot L^{-1}$ to 14.7 $g \cdot L^{-1}$, the bioconversion duration from 55.8 ± 2.2 h to 88.6 h, and the 3-HP production yield from 0.8 ± 0.1 $g_{3\text{-}HP} \cdot g_{CDW}^{-1}$ to 2.0 $g_{3\text{-}HP} \cdot g_{CDW}^{-1}$. In addition, the specific 3-HP production rate was improved from 14.0 ± 2.5 $mg_{3\text{-}HP} \cdot g_{CDW}^{-1} \cdot h^{-1}$ to 22.5 $mg_{3\text{-}HP} \cdot g_{CDW}^{-1} \cdot h^{-1}$. As the cell concentration at the end of the growth phase (3.0 $g_{CDW} \cdot L^{-1}$) was similar to that of the reference condition (3.3 ± 0.3 $g_{CDW} \cdot L^{-1}$), this improvement could be explained by a better ability of each cell to produce 3-HP from glycerol. This was supported by the higher ratio between 3-HP production to enzymatically-active cells determined by flow cytometry, which increased from 0.98 ± 0.21 $g_{3\text{-}HP}.g_{viable\ cells}^{-1}$ in the reference condition to 2.05 $g_{3\text{-}HP}.g_{viable\ cells}^{-1}$ in the optimized recipe.

In conclusion, the influence of various growth conditions was assessed on the growth efficiency and on the ability of resting cells of *L. reuteri* DSM 17938 to produce 3-HP at a given glycerol feeding rate of 0.5 $g \cdot h^{-1}$. The implementation of a Plackett and Burman experimental design

enabled 11 factors to be tested. The supplementation of MRS medium with glucose and the use of a higher temperature (i.e. 37 °C) induced a greater cell quantity at the end of the growth phase. Meanwhile, the addition of phytone peptone, Tween 80, 1,2-PDO, betaine with KCl and the use of a suboptimal temperature together with an optimal pH, were recognized as options to improve the bioconversion duration and the 3-HP production yield. The best set of conditions has been validated as it enhanced the 3-HP titer (+ 25 %), the 3-HP production yield (+ 150 %) and the 3-HP specific production rate (+ 61 %).

With the aim of further improving the 3-HP bioproduction by *L. reuteri* DSM 17938, the environmental conditions during the bioconversion step will have to be optimized, together with avoiding 3-HPA accumulation. Finally, in order to better understand the cellular modifications that are linked to the improvement of 3-HP bioproduction, a more in-depth analysis of the physiological state of the cells may be done, mainly by using omic methods.

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References

1. **Lüthi-Peng, Q., Schärer, S., and Puhan, Z.:** Production and stability of 3-hydroxypropionaldehyde in *Lactobacillus reuteri*, Applied Microbiology and Biotechnology **60**, 73-80 (2002).
2. **Gokarn, R. R., Selifonova, O. V., Jessen, H. J., Gort, S. J., Selmer, T., and Buckel, W.:** 3-Hydroxypropionic acid and other organic compounds **US7186541B2** 186–541 (2007).

3. **Tingirikari, J. M. R., Ahmed, S., and Yata, V. K.:** Chapter 6, 3-hydroxy-propionic acid, pp. 101-117, in: Kaur Brar, S., Jyoti Sarma, S., and Pakshirajan, K. (Ed.), Platform Chemical Biorefinery. Elsevier, Amsterdam (2016).
4. **Bozell, J. J. and Petersen, G. R.:** Technology development for the production of biobased products from biorefinery carbohydrates - the US Department of Energy's "Top 10" revisited, Green Chemistry, **12**, 539-554 (2010).
5. **Chavez-Gonzalez, M. L., Balagurusamy, N., and Aguilar, C. N.:** Advances in Food Bioproducts and Bioprocessing Technologies, pp. 594. CRC Press (2019).
6. **Matsakas, L., Hrůzová, K., Rova, U., and Christakopoulos, P.:** Biological production of 3-hydroxypropionic acid: An update on the current status, Fermentation, **4**, 13-34 (2018).
7. **de Fouchécour, F., Sánchez-Castañeda, A.-K., Saulou-Bérion, C., and Spinnler, H. E.:** Process engineering for microbial production of 3-hydroxypropionic acid, Biotechnology Advances, **36**, 1207-1222 (2018).
8. **Chen, L., Gu, Q., Li, P., Chen, S., and Li, Y.:** Genomic analysis of *Lactobacillus reuteri* WHH1689 reveals its probiotic properties and stress resistance, Food Science & Nutrition, **7**, 844-857 (2019).
9. **Jiang, X., Meng, X., and Xian, M.:** Biosynthetic pathways for 3-hydroxypropionic acid production, Applied Microbiology and Biotechnology, **82**, 995-1003 (2009).
10. **Santos, F., Teusink, B., Molenaar, D., van Heck, M., Wels, M., Sieuwerts, S., de Vos, W. M., and Hugenholtz, J.:** Effect of amino acid availability on vitamin B12 production in *Lactobacillus reuteri*, Applied and Environmental Microbiology, **75**, 3930-3936 (2009).
11. **Lindlbauer, K. A., Marx, H., and Sauer, M.:** 3-hydroxypropionaldehyde production from crude glycerol by *Lactobacillus diolivorans* with enhanced glycerol uptake, Biotechnology for Biofuels, **10**, 295-295 (2017).

12. **Dishisha, T., Pereyra, L. P., Pyo, S.-H., Britton, R. A., and Hatti-Kaul, R.:** Flux analysis of the *Lactobacillus reuteri* propanediol utilization pathway for production of 3-hydroxypropionaldehyde, 3-hydroxypropionic acid and 1,3-propanediol from glycerol, Microbial Cell Factories, **13**, 76-87 (2014).
13. **Talarico, T. L., Axelsson, L. T., Novotny, J., Fiuzat, M., and Dobrogosz, W. J.:** Utilization of glycerol as a hydrogen acceptor by *Lactobacillus reuteri*: Purification of 1,3-propanediol: NAD^+ oxidoreductase, Applied and Environmental Microbiology, **56**, 943-948 (1990).
14. **Sabet-Azad, R., Linares-Pastén, J. A., Torkelson, L., Sardari, R. R., and Hatti-Kaul, R.:** Coenzyme A acylating propionaldehyde dehydrogenase (PduP) from *Lactobacillus reuteri*: Kinetic characterization and molecular modeling, Enzyme and Microbial Technology, **53**, 235-242 (2013).
15. **Chen, L. and Hatti-Kaul, R.:** Exploring *Lactobacillus reuteri* DSM20016 as a biocatalyst for transformation of longer chain 1,2-diols: Limits with microcompartment, PLoS One, **12**, e0185734 (2017).
16. **Dishisha, T., Pyo, S.-H., and Hatti-Kaul, R.:** Bio-based 3-hydroxypropionic-and acrylic acid production from biodiesel glycerol via integrated microbial and chemical catalysis, Microbial Cell Factories, **14**, 200-211 (2015).
17. **Zabed, H. M., Zhang, Y., Guo, Q., Yun, J., Yang, M., Zhang, G., and Qi, X.:** Co-biosynthesis of 3-hydroxypropionic acid and 1,3-propanediol by a newly isolated *Lactobacillus reuteri* strain during whole cell biotransformation of glycerol, Journal of Cleaner Production, **226**, 432-442 (2019).
18. **Burgé, G., Saulou-Bérion, C., Moussa, M., Allais, F., Athes, V., and Spinnler, H.:** Relationships between the use of Embden Meyerhof pathway (EMP) or Phosphoketolase pathway (PKP) and lactate production capabilities of diverse *Lactobacillus reuteri* strains, Journal of Microbiology, **53**, 702-710 (2015).

19. **Arsköld, E., Lohmeier-Vogel, E., Cao, R., Roos, S., Rådström, P., and van Niel, E. W. J.:** Phosphoketolase pathway dominates in *Lactobacillus reuteri* ATCC 55730 containing dual pathways for glycolysis, Journal of Bacteriology, **190**, 206-212 (2008).
20. **Alazzeh, A. Y., Ibrahim, S. A., Song, D., Shahbazi, A., and AbuGhazaleh, A. A.:** Carbohydrate and protein sources influence the induction of α- and β-galactosidases in *Lactobacillus reuteri*, Food Chemistry, **117**, 654-659 (2009).
21. **Couvreur, J., Teixeira, A., Allais, F., Spinnler, H.-E., Saulou-Bérion, C., and Clément, T.:** Wheat and sugar beet coproducts for the bioproduction of 3-hydroxypropionic acid by *Lactobacillus reuteri* DSM17938, Fermentation, **3**, 32-44 (2017).
22. **Ju, J.-H., Wang, D., Heo, S.-Y., Kim, M.-S., Seo, J.-W., Kim, Y.-M., Kim, D.-H., Kang, S.-A., Kim, C.-H., and Oh, B.-R.:** Enhancement of 1,3-propanediol production from industrial by-product by *Lactobacillus reuteri* CH53, Microbial Cell Factories, **19**, 6-16 (2020).
23. **Ichinose, R., Fukuda, Y., Yamasaki-Yashiki, S., and Katakura, Y.:** Suppression of lactate production of *Lactobacillus reuteri* JCM1112 by co-feeding glycerol with glucose, Journal of Bioscience and Bioengineering, **129**, 110-115 (2020).
24. **Atilola, O. A., Gyawali, R., Aljaloud, S. O., and Ibrahim, S. A.:** Use of phytone peptone to optimize growth and cell density of *Lactobacillus reuteri*, Foods, **4**, 318-327 (2015).
25. **Ayad, A. A., Gad El-Rab, D. A., Ibrahim, S. A., and Williams, L. L.:** Nitrogen sources effect on *Lactobacillus reuteri* growth and performance cultivated in date palm (*Phoenix dactylifera L.*) by-products, Fermentation, **6**, 64-74 (2020).
26. **Lozo, J., Begovic, J., Jovcic, B., Golić, N., and Topisirovic, L.:** Effect of methionine and cysteine deprivation on growth of different natural isolates of *Lactobacillus* spp. in chemically defined media, Archives of Biological Sciences, **60**, 509-517 (2008).

27. **Turner, M. S., Woodberry, T., Hafner, L. M., and Giffard, P. M.:** The *bsp*A locus of *Lactobacillus fermentum* BR11 encodes an L-cystine uptake system, Journal of Bacteriology, **181**, 2192-2198 (1999).
28. **Reitermayer, D., Kafka, T. A., Lenz, C. A., and Vogel, R. F.:** Interrelation between Tween and the membrane properties and high pressure tolerance of *Lactobacillus plantarum*, BMC Microbiology, **18**, 72-84 (2018).
29. **Burgé, G., Saulou-Bérion, C., Moussa, M., Pollet, B., Flourat, A., Allais, F., Athès, V., and Spinnler, H.-E.:** Diversity of *Lactobacillus reuteri* strains in converting glycerol into 3-hydroxypropionic acid, Applied Biochemistry and Biotechnology, **177**, 923-939 (2015).
30. **Sriramulu, D. D., Liang, M., Hernandez-Romero, D., Raux-Deery, E., Lünsdorf, H., Parsons, J. B., Warren, M. J., and Prentice, M. B.:** *Lactobacillus reuteri* DSM 20016 produces cobalamin-dependent diol dehydratase in metabolosomes and metabolizes 1,2-propanediol by disproportionation, Journal of Bacteriology, **190**, 4559-4567 (2008).
31. **Louesdon, S., Charlot-Rougé, S., Juillard, V., Tourdot-Maréchal, R., and Béal, C.:** Osmotic stress affects the stability of freeze-dried *Lactobacillus buchneri* R1102 as a result of intracellular betaine accumulation and membrane characteristics, Journal of Applied Microbiology, **117**, 196-207 (2014).
32. **Polak-Berecka, M., Waśko, A., Kordowska-Wiater, M., Podleśny, M., Zdzislaw, T., and Kubik-Komar, A.:** Optimization of medium composition for enhancing growth of *Lactobacillus rhamnosus* PEN using response surface methodology, Polish Journal of Microbiology, **59**, 113-118 (2010).
33. **Garro, M. S., de Valdez, G. F., and de Giori, G. S.:** Temperature effect on the biological activity of *Bifidobacterium longum* CRL 849 and *Lactobacillus fermentum* CRL 251 in pure and mixed cultures grown in soymilk, Food Microbiology, **21**, 511-518 (2004).

34. **Kandler, O., Stetter, K.-O., and Köhl, R.:** *Lactobacillus reuteri* sp. nov., a new species of heterofermentative lactobacilli, Zentralblatt für Bakteriologie: I. Abt. Originale C: Allgemeine, Angewandte und ökologische Mikrobiologie, **1**, 264-269 (1980).
35. **El-Ziney, M. G.:** Molecular and Probiotic Characterizations of *Lactobacillus reuteri* DSM 12246 and Impact of pH on Biomass and Metabolic Profile in Batch-Culture, Advances in Microbiology, **8**, 13-30 (2018).
36. **Hernández, A., Larsson, C. U., Sawicki, R., van Niel, E. W. J., Roos, S., and Håkansson, S.:** Impact of the fermentation parameters pH and temperature on stress resilience of *Lactobacillus reuteri* DSM 17938, AMB Express, **9**, 66-72 (2019).
37. **Kristjansdottir, T., Bosma, E. F., Branco dos Santos, F., Özdemir, E., Herrgård, M. J., França, L., Ferreira, B., Nielsen, A. T., and Gudmundsson, S.:** A metabolic reconstruction of *Lactobacillus reuteri* JCM 1112 and analysis of its potential as a cell factory, Microbial Cell Factories, **18**, 186-205 (2019).
38. **Ricci, M. A., Russo, A., Pisano, I., Palmieri, L., de Angelis, M., and Agrimi, G.:** Improved 1,3-propanediol synthesis from glycerol by the robust *Lactobacillus reuteri* strain DSM 20016, Journal of Microbiology and Biotechnology, **25**, 893-902 (2015).
39. **Rault, A., Béal, C., Ghorbal, S., Ogier, J.-C., and Bouix, M.:** Multiparametric flow cytometry allows rapid assessment and comparison of lactic acid bacteria viability after freezing and during frozen storage, Cryobiology, **55**, 35-43 (2007).
40. **Plackett, R. L. and Burman, J. P.:** The design of optimum multifactorial experiments, Biometrika, **33**, 305-325 (1946).
41. **Papadimitriou, K., Alegría, Á., Bron, P. A., de Angelis, M., Gobbetti, M., Kleerebezem, M., Lemos, J. A., Linares, D. M., Ross, P., Stanton, C., and other seven authors:** Stress physiology of lactic acid bacteria, Microbiology and molecular biology reviews: MMBR, **80**, 837-890 (2016).

42. **van Bokhorst-van de Veen, H., Abee, T., Tempelaars, M., Bron, P. A., Kleerebezem, M., and Marco, M. L.:** Short-and long-term adaptation to ethanol stress and its cross-protective consequences in *Lactobacillus plantarum*, Applied Environmental Microbiology, **77**, 5247-5256 (2011).

43. **Cleusix, V., Lacroix, C., Vollenweider, S., Duboux, M., and Le Blay, G.:** Inhibitory activity spectrum of reuterin produced by *Lactobacillus reuteri* against intestinal bacteria, BMC Microbiology, **7**, 101-110 (2007).

44. **Schaefer, L., Auchtung, T. A., Hermans, K. E., Whitehead, D., Borhan, B., and Britton, R. A.:** The antimicrobial compound reuterin (3-hydroxypropionaldehyde) induces oxidative stress via interaction with thiol groups, Microbiology, **156**, 1589-1599 (2010).

45. **Dishisha, T., Sabet-Azad, R., Arieta, V., and Hatti-Kaul, R.:** *Lactobacillus reuteri* NAD(P)H oxidase: Properties and coexpression with propanediol-utilization enzymes for enhancing 3-hydroxypropionic acid production from 3-hydroxypropionaldehyde, Journal of Biotechnology, **289**, 135-143 (2019).

46. **Duar, R. M., Lin, X. B., Zheng, J., Martino, M. E., Grenier, T., Pérez-Muñoz, M. E., Leulier, F., Gänzle, M., and Walter, J.:** Lifestyles in transition: Evolution and natural history of the genus *Lactobacillus*, FEMS Microbiology Reviews, **41**, S27-S48 (2017).

47. **Stuart, M. R., Chou, L. S., and Weimer, B. C.:** Influence of carbohydrate starvation and arginine on culturability and amino acid utilization of *Lactococcus lactis* subsp. *lactis*, Applied and Environmental Microbiology, **65**, 665-673 (1999).

48. **Champomier Vergès, M.-C., Zuñiga, M., Morel-Deville, F., Pérez-Martínez, G., Zagorec, M., and Ehrlich, S. D.:** Relationships between arginine degradation, pH and survival in *Lactobacillus sakei*, FEMS Microbiology Letters, **180**, 297-304 (1999).

659 49. **Béal, C., Fonseca, F., and Corrieu, G.:** Resistance to freezing and frozen storage of
660 *Streptococcus thermophilus* is related to membrane fatty acid composition, Journal of
661 Dairy Science, **84**, 2347-2356 (2001).
662 50. **Kets, E. P. W. and de Bont, J. A. M.:** Protective effect of betaine on survival of
663 *Lactobacillus plantarum* subjected to drying, FEMS Microbiology Letters, **116**, 251-256
664 (1994).

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Table S1. Experimental factors and their levels in the Plackett and Burman experimental design

Experiment code	Experimental factors										
	Additional glucose ($g \cdot L^{-1}$)	Additional yeast extract ($g \cdot L^{-1}$)	Phytone peptone ($g \cdot L^{-1}$)	1,2-propanediol ($g \cdot L^{-1}$)	Cysteine ($g \cdot L^{-1}$)	Betaine ($g \cdot L^{-1}$) and KCl ($g \cdot L^{-1}$)	Tween 80 ($g \cdot L^{-1}$)	Vitamin B12 ($mg \cdot L^{-1}$)	Temperature (°C)	pH	Base type (and concentration, in $mol \cdot L^{-1}$)
T1	20	0	0	0	0	0 and 0	1	0	37	6.0	NH_4OH (14.8)
T2	50	0	25	0	0	0 and 0	5	0.1	33	6.0	NaOH (8.75)
T3	50	25	0	3	0	0 and 0	1	0.1	37	5.5	NH_4OH (14.8)
T4	20	25	25	0	1	0 and 0	1	0	37	5.5	NaOH (8.75)
T5	50	0	25	3	0	0.234 and 0.745	1	0	33	5.5	NaOH (8.75)
T6	50	25	0	3	1	0 and 0	5	0	33	6.0	NaOH (8.75)
T7	50	25	25	0	1	0.234 and 0.745	1	0.1	33	6.0	NH_4OH (14.8)
T8	20	25	25	3	0	0.234 and 0.745	5	0	37	6.0	NH_4OH (14.8)
T9	20	0	25	3	1	0 and 0	5	0.1	33	5.5	NH_4OH (14.8)
T10	20	0	0	3	1	0.234 and 0.745	1	0.1	37	6.0	NaOH (8.75)
T11	50	0	0	0	1	0.234 and 0.745	5	0	37	5.5	NH_4OH (14.8)
T12	20	25	0	0	0	0.234 and 0.745	5	0.1	33	5.5	NaOH (8.75)

Table S2. Data characterizing *L. reuteri* DSM 17938 growth and glycerol bioconversion performances

	Cell concentration ($g_{CDW} \cdot L^{-1}$)	Top 3-HP titer ($g \cdot L^{-1}$)	Top 1,3-PDO titer ($g \cdot L^{-1}$)	Total 3-HP produced (g)	Total 1,3-PDO produced (g)	3-HP production yield ($g_{3\text{-}HP} \cdot g_{CDW}^{-1}$)	Bioconversion duration (h)
T1a	3.4	11.2	9.4	12.4	10.4	0.76	57.7
T1b	3.0	11.9	9.0	13.7	10.4	0.90	53.4
T1c	3.6	12.3	10.0	14.1	11.5	0.68	56.2
T2	3.0	10.9	10.0	12.4	11.3	0.60	51.4
T3	4.8	9.9	10.4	11.1	11.6	0.42	52.2
T4	3.2	11.0	10.0	13.3	12.1	0.88	63.2
T5	5.1	13.9	14.0	18.9	19.0	0.61	92.3
T6	5.7	12.7	12.8	16.1	16.1	0.50	75.6
T7	4.5	9.9	11.3	11.9	13.5	0.35	64.3
T8	3.0	14.7	10.9	19.6	15.7	1.99	88.6
T9	4.5	11.7	10.8	14.3	13.2	0.68	58.8
T10	2.4	10.1	8.0	11.0	8.7	0.83	44.1
T11	3.8	12.9	12.7	16.3	16.0	0.63	72.7
T12	3.5	9.8	9.2	11.1	10.4	0.68	50.1